

Time Course Changes of Oxidative Stress Markers in a Rat Experimental Glaucoma Model

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PURPOSE. To evaluate the relationship between oxidative stress markers and increased intraocular pressure in experimental glaucoma.

METHODS. In vivo chemiluminescence (CL), total antioxidant capacity (TRAP), nitrite concentration (NC), and lipid peroxidation markers (TBARS) were evaluated. Wistar rats ($n = 18$ for each time point) underwent operation, and two episcleral veins were cauterized.

RESULTS. Decreases of 22%, 35%, and 27% at 7, 15, and 30 days and an increase of 22% at 60 days in CL were observed in glaucomatous eyes. In optic nerve, TBARS values were 6.9 ± 0.5 nmol/mg protein (7 days), 9.4 ± 0.4 nmol/mg protein (15 days), 18.0 ± 1.2 nmol/mg protein (30 days), and 43.1 ± 5.3 nmol/mg protein (60 days) (control, 6.2 ± 0.4 nmol/mg protein; $P < 0.001$). NC was 37.0 ± 1.8 μ M (7 days), 31.4 ± 1.2 μ M (15 days), 39.6 ± 1.3 μ M (30 days), and 40.0 ± 1.3 μ M (60 days) (control, 21.1 ± 1.7 μ M; $P < 0.001$). In glaucomatous vitreous humor, TRAP decreased by 42% at 15 days and 78% at 60 days (control, 414 ± 29 μ M; $P < 0.001$). In glaucomatous aqueous humor, TRAP values were 75 ± 7 μ M (7 days), 54 ± 4 μ M (15 days), 25 ± 4 μ M (30 days), and 50 ± 3 μ M (60 days) (control, 90 ± 10 μ M; $P < 0.001$).

CONCLUSIONS. Reactive species were increased in glaucoma, as evidenced by the increases in CL, TBARS, and NC. The decrease in the antioxidant levels may be a consequence of an increase in oxidative processes. (*Invest Ophthalmol Vis Sci* 2010;51:4635–4640) DOI:10.1167/iops.09-5044

Glaucoma is a disease characterized by a progressive and typical degeneration of optic nerve head and visual field damage.^{1,2} Elevated intraocular pressure (IOP) is the most important known risk factor for the development of glaucomatous optic nerve damage. Several concomitant factors including ischemia,³ obstruction of axoplasmic flow,⁴ deprivation of trophic factors,⁵ and excitotoxicity⁶ and oxidative stress^{7,8} may contribute to glaucomatous optic neuropathy.

Oxidative stress can be defined as an increase over physiological values in the intracellular concentrations of reactive oxygen and nitrogen species.⁹ Evidence of oxidative and nitra-

tive processes was found in several ocular disorders in terms of activity of antioxidant enzymes, levels of low-molecular weight antioxidants, and markers of lipid peroxidation.^{10,11} Moreover, it has been reported that nitric oxide (NO) may be an important mediator in retinal ganglion cell death in glaucoma.¹²

Among oxidative stress markers, organ chemiluminescence seems to afford a noninvasive assay that integratively measures the rate of formation of excited species, mostly singlet oxygen, through the measurement of light emission.

Light emission from in situ organs is related to the in vivo steady state concentration of reactive oxygen species. An increased chemiluminescence level reflects an increased intracellular concentration of excited species, singlet oxygen, excited carbonyls, and peroxy radicals. Increased levels of chemiluminescence indicated the occurrence of oxidative stress.¹³ Chemiluminescence is a noninvasive, nondestructive assay that can be useful in monitoring cellular damage in glaucomatous eyes.¹⁴

An experimental glaucoma model in rats was performed to evaluate the time course changes in oxidative stress markers. This model induced high intraocular pressure and optic nerve head damage. It appeared to mimic features of primary open angle glaucoma; therefore, it may be useful to understand the time course of this ocular disease.

The purpose of the present study was to evaluate the correlation between a time period of increased intraocular pressure and the changes in oxidative stress markers in a rat experimental glaucoma model.

To assess the occurrence of oxidative stress, the following markers were evaluated: in vivo chemiluminescence of the eye surface, total antioxidant capacity (TRAP) in the aqueous and vitreous humor, and nitrite concentration and markers of lipid peroxidation in the optic nerve head.

MATERIALS AND METHODS

Experimental Glaucoma Model

The chronic ocular hypertension model following episcleral venous occlusion in rats was used.¹⁵ Female Wistar rats (age, 3 months; $n = 108$) weighing 250 to 300 g underwent surgery under a microscope with a coaxial light. Animals were anesthetized with ketamine hydrochloride (50 mg/kg) and xylazine hydrochloride (0.5 mg/kg) administered intraperitoneally.

A specially designed small lid speculum was used to retract the eyelids. One drop of 0.5% proparacaine hydrochloride (Alcon Laboratories, Buenos Aires, Argentina) was instilled. Vannas scissors and a conjunctiva forceps were used to open the conjunctiva and expose the limbal veins. A cyclodialysis spatula was used to gently lift the vein from the underlying sclera, and an ophthalmic cautery was used to cauterize the vein. Care was taken not to damage the sclera. Two of the large veins of the left eye were cauterized using this method for the glaucoma group ($n = 9$ for each time point). Retraction without

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bleeding was noted after cauterization. Only one eye per animal was used for the experiment.

A sham operation without cauterization of the vessels was performed in the left eye of the control group ($n = 9$ for each time point). The right eye was used only as a control for ophthalmological examination in both groups.

Rats were housed in a standard animal room in a 12-hour light/12-hour dark cycle and were fed food and water ad libitum under controlled temperature conditions ($21^{\circ}\text{C} \pm 2^{\circ}\text{C}$) and humidity. After different periods of time (0, 7, 15, 30, 45, 60 days), eyes were enucleated under dim light immediately after anesthesia, and aqueous humor, vitreous humor, and retinas were carefully removed. Vitreous and aqueous humor were collected in a syringe under a surgical microscope, and the retinas were detached by blunt dissection. Immediately after dissection, the optic nerve heads were homogenized.

All animal procedures were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Intraocular Pressure Assessment

Intraocular pressure was measured with a tonometer (XL Mentor; Tono-Pen, Norwell, MA). All IOP determinations were performed in unanesthetized rats, as previously described.¹⁶ Animals were wrapped in a towel and held to make the readings. Eyelids were gently opened, with care taken not to compress the eyes, and the tonometer (XL Mentor; Tono-Pen) was applied to the cornea. Fifteen readings were taken and averaged for each record. The instrument was applied firmly and perpendicularly to the cornea to obtain the readings. IOP measure-

ments were performed before and after the procedure at 0, 7, 15, 30, 45, and 60 days. Results were expressed as millimeters of mercury.

In Vivo Chemiluminescence

Spontaneous chemiluminescence (CL) was measured with a photon counter (Johnson Research Foundation, University of Pennsylvania, Philadelphia, PA) specially adapted for organ CL, as previously described. A photomultiplier (EMI 9658; EMI-Gencom, Plainview, NY) responsive from 300 to 900 nm and cooled at -20°C with an applied potential of -1.4 kW was used. The phototube output was connected to an amplifier-discriminator adjusted to a single photon counting that was, in turn, connected to both a frequency counter and a recorder. Efficient light collection and isolation from the sample were established by using a polymethyl methacrylate rod as an optical coupler placed in front of the exposed organ in situ (Fig. 1).

Rats were anesthetized with ketamine hydrochloride (50 mg/kg) and xylazine hydrochloride (0.5 mg/kg), and the eye was exposed. CL is expressed as counts per second of organ surface (cps/cm^2).^{13,14}

Total Reactive Antioxidant Potential

A method based on the capacity of the sample to scavenge luminol luminescence induced by thermolysis of 2,2'-azobis-amidinopropane (ABAP) was used as previously described.¹⁵ ABAP is a source of free radicals that react with luminol, yielding chemiluminescence. The addition of samples to the reaction medium consisting of 100 mM phosphate buffer (pH 7.40), 20 mM (ABAP), and 40 μM luminol decreases chemiluminescence to basal levels for a period (induction

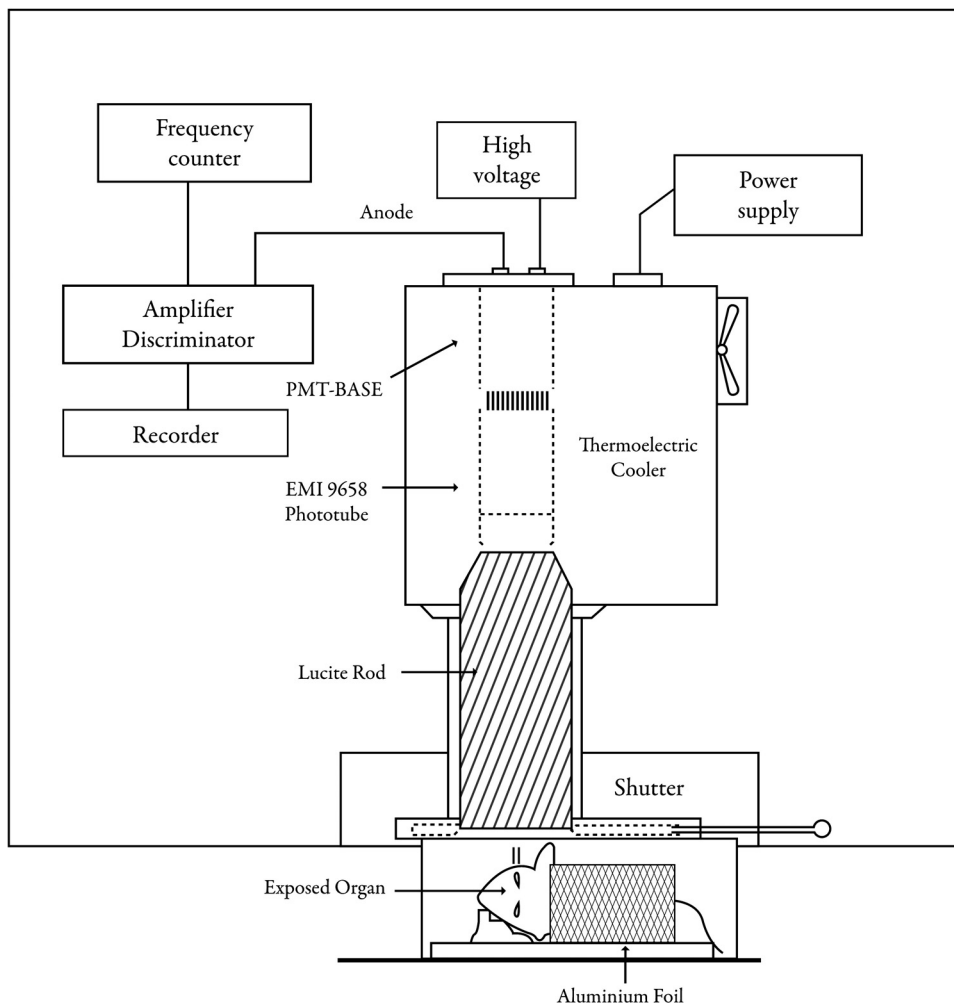


FIGURE 1. Scheme of the photon counter used for the measurement of in vivo eye chemiluminescence.

time) proportional to the total load of antioxidants present in the aqueous or in the vitreous humor. Chemiluminescence was measured using a liquid scintillation counter in the out-of-coincidence mode. The system is calibrated with different concentrations (0.25–0.50 μM) of a vitamin E water-soluble analog (Trolox; Hoffman-LaRoche, Basel, Switzerland). Comparison of the induction time after the addition of the analog and the aqueous or vitreous humor allowed calculation of the total antioxidant capacity (TRAP) as the equivalent of the analog concentration necessary to produce the same induction time. Results were expressed in micromolar.¹⁷

Nitrite Concentration

The nitrite concentration was measured using a spectrophotometric method based on the Griess reaction. Samples were mixed with 1% sulfanilamide and 0.1% naphthyl-ethylenediamine, which were allowed to react at room temperature for 10 minutes. The nitrite concentration was determined by measuring the absorbance at 550 nm in comparison with standard solutions of sodium nitrite.¹⁸

Thiobarbituric Acid Reactive Substances

Thiobarbituric acid reactive substances (TBARS) were determined in the optic nerve head homogenates with the use of a spectrophotometric method based on the 2-thiobarbituric acid reaction.¹⁹ Each retina and the optic nerve head were homogenized in 100 mM sodium phosphate plus 120 mM potassium chloride buffer (pH 7.40) and were centrifuged at 900g for 5 minute at 4°C. TBARS was detected at 532 nm, using a spectrophotometer (Hitachi, Tokyo, Japan). The molar extinction coefficient ($1.54 \times 10^5 \text{ M}^{-1} \cdot \text{cm}^{-1}$) allows calculating the concentration of malondialdehyde. Results were expressed in nmol/mg protein. Protein content was determined by the method of Lowry et al.²⁰

Chemicals

All chemicals were purchased from Sigma-Aldrich Chemical (St. Louis, MO).

Statistical Analysis

Statistical calculations were performed (InStat statistical package for Windows; GraphPad, San Diego, CA). Data are expressed as mean \pm SEM. Statistical significance of the differences between the groups was calculated by the two-tailed unpaired Student's *t*-test, and $P < 0.01$ indicated a statistically significant difference.

RESULTS

Figure 2 shows the average IOP of rats with glaucoma during 0, 7, 15, 30, 45, and 60 days. At all these times, a significant increase in IOP was observed in the left eyes compared with the control eyes. Elevated IOP is the most important risk factor

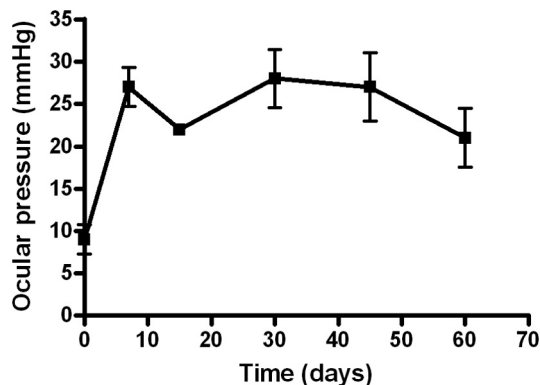


FIGURE 2. Intraocular pressure values of rats at 0, 7, 15, 30, 45, and 60 days. Surgery was performed at time 0.

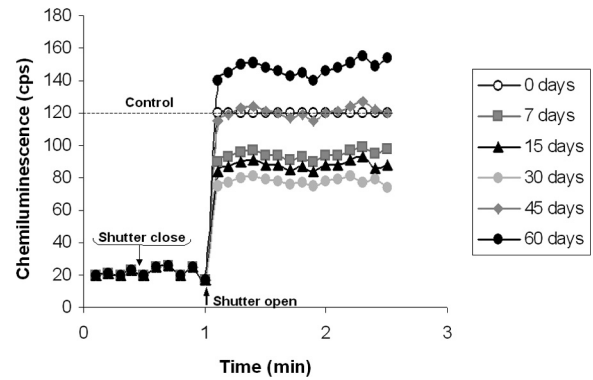


FIGURE 3. Traces of in vivo chemiluminescence obtained of the eye at different times. Surgery was performed at time 0. Baseline was recorded when the shutter was closed.

in the progression of glaucomatous damage. All animals, without exception, responded with an increase in IOP after the development of experimental glaucoma surgery. No differences in the IOP of control eyes were detected during the experimental period. A significant increase in IOP compared with that in control eyes was observed 7 days after surgery.

Figure 3 shows a record of spontaneous chemiluminescence of the eye surface. On opening the shutter, the chemiluminescence of 0, 7, 15, 30, 45, and 60 days was recorded. At 0 days the chemiluminescence reached 120 cps. The initial traces corresponded to the background emission (20 cps baseline line), and the upper traces corresponded to the measurements after the shutter was opened for control (120 cps) and for each time point of hypertensive eyes. Maximal emission was reached at 60 days of the development of glaucoma model (160 cps). Control eyes did not change the light emission among these time points.

An analysis of spontaneous eye surface luminescence at 0, 7, 15, 30, 45, and 60 days was shown in Figure 4. Initially the luminescence showed a decrease in the eye photoemission by 22%, 35%, and 27%, respectively, at 7, 15, and 30 days after the surgery. An increase of 22% in light emission was observed at 60 days. Chemiluminescence at 0 days was considered 100% of light emission, and results were compared with this value.

Aqueous humor samples were compared regarding the alterations in the levels of nonenzymatic antioxidants measuring TRAP. A significant decrease in levels of nonenzymatic antioxidants was found in the aqueous humor of hypertensive eyes after 15 days of the surgery. A significant decrease in TRAP values was observed at 30 days compared with 15 days; on the

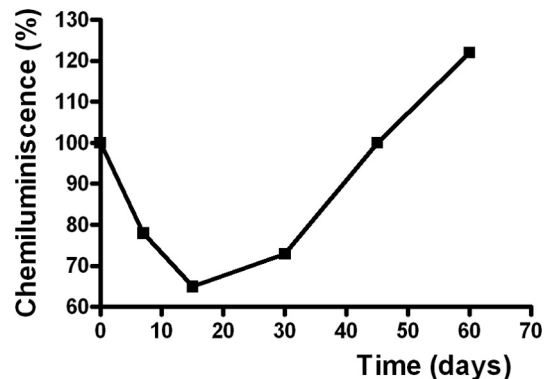


FIGURE 4. Analysis of spontaneous chemiluminescence percentage at different times. Surgery was performed at time 0.

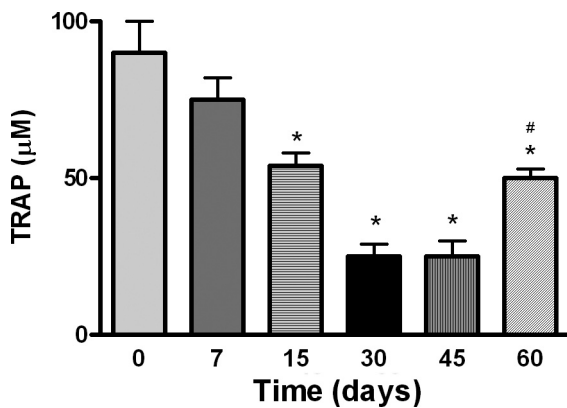


FIGURE 5. TRAP in the aqueous humor at different times. Data are mean \pm SEM. * $P < 0.001$ versus time 0. # $P < 0.05$ versus time 30 and 45 days. Surgery was performed at time 0.

other hand, the levels of this parameter did not change compared with 45 days. At 60 days, TRAP levels were significantly higher than at 30 or 45 days ($P < 0.05$), whereas, these levels were significantly lower than at 7 days of treatment (Fig. 5).

Figure 6 shows the average of TRAP values in the vitreous humor of rats at 0, 7, 15, 30, 45, and 60 days after surgery. A significant decrease in the levels of nonenzymatic antioxidants was found in the vitreous humor of hypertensive eyes after 7 days of surgery. No significant changes were observed in the levels of TRAP at 7 days compared with 15 days. Between 15 and 60 days there was a significant and progressive decrease in the levels of nonenzymatic antioxidants over the experimental period. The percentage of decrease was 42% for 15 days and 78% for 60 days compared with control eyes.

TBARS level as an index of lipid peroxidation was assessed in the optic nerve head from the hypertensive rats or from sham rats for the same periods (Fig. 7). At 30 days of surgery, TBARS levels were significantly higher (200%) in hypertensive rats than in sham rats (6.24 ± 0.42 nmol/mg protein; $P < 0.001$). A further significant increase was observed at 45 and 60 days (300% and 600%, respectively; $P < 0.001$). No significant changes were observed at 7 and 15 days compared with control value (6.24 ± 0.42 nmol/mg protein).

Nitrite levels in the optic nerve head were represented at different times of surgery in Figure 8. A significant increase of nitrite concentration in the optic nerve head was found 7 days after surgery. This increase was maintained until 60 days of treatment. There was no significant difference between nitrite levels at 7, 15, 30, and 45 days.

Figure 9 showed a negative correlation between TRAP and TBARS levels. The levels of lipid peroxidation products were

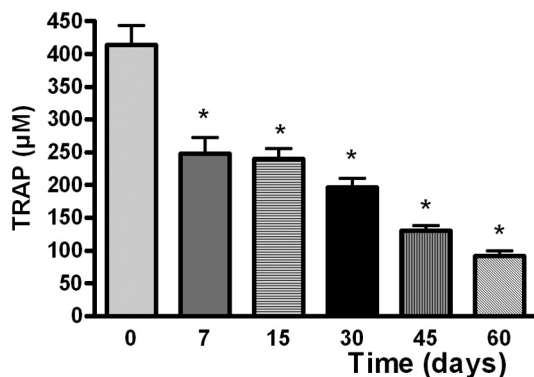


FIGURE 6. TRAP in the vitreous humor at different times. Data are mean \pm SEM. * $P < 0.001$. Surgery was performed at time 0.

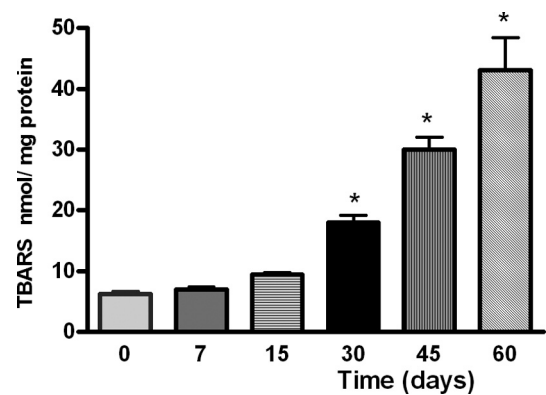


FIGURE 7. Optic nerve head TBARS levels at different times. Data are mean \pm SEM. * $P < 0.001$. Surgery was performed at time 0.

increased while the levels of nonenzymatic antioxidants were decreased in the optic nerve head of rats with glaucoma.

DISCUSSION

This experimental glaucoma model was used to elucidate the role of oxidative stress in this pathologic condition. Previous studies of our group have reported the occurrence of oxidative stress on the aqueous humor of glaucoma patients.^{7,21}

The response of antioxidant defense mechanisms is assessed by the measurements of spontaneous chemiluminescence, TBARS, nitric oxide and non-enzymatic antioxidants levels.

Our results show for the first time the spontaneous chemiluminescence of the eye in rats with experimental glaucoma and in control eyes. Chemiluminescence is the emission of radiation resulting from a chemical reaction. Organ chemiluminescence is a method to evaluate signals of oxidative metabolism. This assay is specific and noninvasive for the organ and provides a time course evaluation of peroxidative breakdown of lipids. The termination reaction of peroxy radicals and singlet oxygen yields excited states and chemiluminescence in parallel with malondialdehyde production and conjugated lipid dienes. Oxidative stress produced an imbalance between the rate of production of oxy and peroxy radicals and the levels of the antioxidant defenses. In the first 30 days we observed a decrease in relative chemiluminescence, which might have been due in part to the consumption of nonenzymatic antioxidants. Up to 30 days the marked increase in the spontaneous chemiluminescence of the eye appeared to indicate an in-

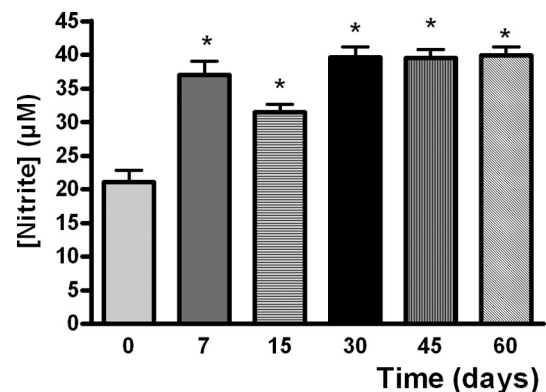


FIGURE 8. Nitrite concentration in the optic nerve head at different times. Data are mean \pm SEM. * $P < 0.001$. Surgery was performed at time 0.

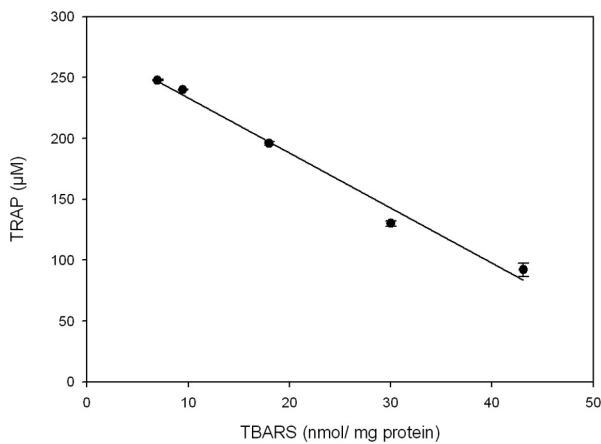


FIGURE 9. Correlation between TRAP and TBARS levels.

crease in the steady state levels of oxidant species, suggesting the occurrence of oxidative stress. Increased eye chemiluminescence was associated with the development of cell injury after oxidative stress that could not be compensated by the antioxidant defenses. Organ chemiluminescence seems to be a useful tool for studying the occurrence of oxidative stress and for evaluating the redox status of the tissues in real time in several pathologic conditions, including glaucoma. Reactive oxygen and nitrogen species were increased in glaucoma, as could be evidenced by the increases in chemiluminescence, nitrite levels, and lipid peroxidation.

Oxidative stress is thought to contribute to the pathophysiology of many neurodegenerative diseases. The retina contains large amounts of polyunsaturated fatty acids and thus could be susceptible to oxidation by free radicals. Furthermore, elevated intraocular pressure or vascular diseases altered blood flow. The consequent decrease in perfusion of the retina and optic nerve head can cause ischemia, which affects retinal ganglion cell survival. Reactive oxygen species are involved in signaling retinal ganglion cell death by acting as a second messenger, modulating protein function, or both.²² Oxidative stress induces the dysfunction of retinal ganglion cells and may contribute to spreading neuronal damage. Chronic elevation of IOP demonstrated significant loss of retinal ganglion cells and has a measurable effect on the redox status.²³

The retina exhibits a distinct susceptibility to oxidative stress because of its enhanced metabolic rate with high levels of oxygen demand and higher lipid content in its membranes. The TBARS levels support this situation; the increase in them was time dependent and correlated with high IOP. In the present study, a significant increase in lipid peroxidation occurred through 15 days after IOP elevation. Previous studies measured TBARS levels in isolated retinas, and a significant increase was found 3 weeks after IOP elevation.²⁴

A negative correlation was found between TRAP and TBARS levels; the levels of lipid peroxidation products were increased while the levels of nonenzymatic antioxidants were decreased in the optic nerve head of rats with glaucoma. Antioxidants are molecules that prevent or reduce the extent of the oxidative destruction of biomolecules. The consumption of endogenous antioxidants, assessed by the decrease in TRAP levels, may be due to an increase in tissue oxidants, as was evidenced by TBARS levels.

Nanning et al.²⁵ demonstrated the release of NO by superoxide. This would result in the formation of peroxyxynitrite that leads to cytotoxic effects in the surrounding cells. In our work, a significant increase of nitrite concentration in the optic nerve head was found 7 days after surgery, and this increase was maintained until 60 days of treatment. Therefore, NO may be a

mediator in ganglion cell death. Siu et al.²⁶ showed that NO levels were increased in retinas after IOP elevation. Neufeld et al.²⁷ reported increased levels of NO synthase (NOS) isoforms in the optic nerve head, and NOS inhibitor provided protection for retinal ganglion cells.²⁸ Conversely, Kasmala et al. (Kasmala LT, et al. *IOVS* 2004; 45: E-Abstract 904) showed that oral administration of an inducible NOS (iNOS) inhibitor did not protect the optic nerve in a rat model. Morrison et al. (Morrison JC, et al. *IOVS* 2003; 44: E-Abstract 2101) have reported that iNOS activity is not elevated in an experimental glaucoma model.

The capacity of NO to induce apoptosis has been documented in astrocytes²⁹ and neuronal cells.³⁰ Further detailed studies are required to evaluate and clarify the role of NO in glaucoma.

The increased chemiluminescence observed at a later stage could be attributed to a decrease in the antioxidant defenses, evidenced by TRAP decreases in aqueous and vitreous humor. Interestingly, a different trend was observed in the time course of TRAP between aqueous humor and vitreous humor at 45 and 60 days, possibly because of a different turnover rate between aqueous and vitreous humor. The levels of lipid peroxidation products and nitric oxide were increased during the development of glaucomatous optic neuropathy.

According to several investigation lines, there is great deal of evidence that oxidative stress may be involved in the development or progression of glaucomatous damage. Moreno et al.³¹ demonstrated a significant decrease in superoxide dismutase and catalase activities, although glutathione peroxidase activity was increased in a glaucoma model after the chronic injection of hyaluronic acid in rats. On the other hand, Ko et al.²⁴ reported a significant increase in superoxide dismutase and catalase activities after 1 week of IOP elevation, and then the activities returned to normal ranges.

Cells usually tolerate mild oxidative stress, resulting in an upregulation of the antioxidant defense system, to restore the antioxidant-oxidant balance. In our study, it seemed possible that the decrease in nonenzymatic antioxidant could overcome the ability of cells to resist oxidative damage. The increased levels of lipid peroxidation products and in vivo chemiluminescence demonstrate this damage.

The relationship between oxidative stress and neurodegeneration is not completely clear. Free radicals can act directly as neurotoxic agents, or they may also function as secondary messengers to spread damage. The impact of oxidative stress in the development of glaucomatous damage could be followed up by analyzing the time course of chemiluminescence of the eye.

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